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Clonal Identities and Multiple Isotype Transcripts in Hematological Diseases Revealed by a Single-Strand Conformation Polymorphism Analysis of the Immunoglobulin Heavy Chain Messenger Signals

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A single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR)-amplified products of immunoglobulin (Ig) heavy chain rearrangements can be used to analyze B cell clonalities and clonal identities of B cells from different samples. However, the usefulness of the PCR-SSCP analysis is not fully assessed in B cell malignancies. For example, we did not know whether the PCR-SSCP method can be used to detect tumor-related clones in peripheral blood of patients with multiple myeloma and Waldenström's macroglobulinemia. In addition, because genomic DNA is used in the PCR-SSCP method, we could obtain no information about the isotype of the expanded B cell clone. In this study, we combined the reverse transcriptase (RT)-PCR of immunoglobulin heavy chain transcripts with an SSCP analysis and thus analyzed eight healthy individuals, five patients with B chronic lymphocytic leukemia, four patients with multiple myeloma and three patients with Waldenström's macroglobulinemia. Clonal B cell populations were detected as discrete bands in the RT-PCR-SSCP analysis that can be readily detected over the background of polyclonal rearrangements. Circulating tumor-related clones were detected in all but one peripheral blood sample from multiple myeloma and Waldenström's macroglobulinemia patients and B cell clones in peripheral blood and bone marrow from these patients showed a similar mobility on SSCP gel. Because the transcripts of different isotypes were separately analyzed, we could thus determine the isotype of B cell clones as well. When monoclonal Igs of different isotypes were detected in the individual samples, we analyzed the relationship of each monoclonal band by excising the band and then further analyzing it by a PCR-SSCP analysis. RT-PCR amplification in conjunction with the SSCP analysis is thus considered to be a useful method to detect and characterize the B cell clones in hematological diseases. Am. J. Hematol. 62:74–81, 1999. © 1999 Wiley-Liss, Inc.

Key words: immunoglobulin variable region; polymerase chain reaction; polymorphism, single-stranded conformational

INTRODUCTION

In progenitor B cells, identified by the presence of CD19 on the surface of the cell and the absence of a cytoplasmic or surface immunoglobulin (Ig) heavy (H) chain, the assembly of the IgH chain variable domain (V) begins with the joining of a DH to a JH gene segment, followed by a rearrangement between the VH gene segment and the intermediate DH-JH join [1,2]. The expression of the cytoplasmic μ chain defines precursor B cells. Such cells can express low levels of surface IgM in association with a pseudo-light chain. The producive rearrangement of a κ or λ light (L) chain leads to the surface

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expression of naive IgM, which thus subjects the B cells to positive and negative selection based on the specificity of its mature antigen receptor [3]. Although such rearrangement stops after the loss of recombinase activity in the bone marrow, further changes in the V gene occur during the immune response in the germinal centers of the lymphoid follicles [4].

The IgH and L chains each contain three complementarity determining regions (CDRs) and four conserved framework areas (FRs) [5]. The CDRs are the most diverse regions of the antibody molecule. In the H chain, two CDRs (CDR1 and CDR2) are encoded by the VH gene segment. The CDR3 is in direct contact with the antigen and is the most variable portion of the Ig molecule [6,7].

The remarkable diversity of CDR3 has been exploited to identify the clonal populations of B cells [8–11]. The polymerase chain reaction (PCR) is advantageous for this purpose because it amplifies the CDR3 sequence in question thousands of times [12]. The PCR product from a polyclonal B cell population shows a broad band when analyzed on agarose or polyacrylamide gels. DNA from B cell lines and B lymphoproliferative disorders shows a discrete band either on native gels or as an increase in one particular band on denaturing gels. The sensitivity of such methods is limited by the background of the identically sized polyclonal IgH chain gene rearrangement. When agarose gels are used for the electrophoretic analysis, it is often difficult to prove that an ethidium bromide band represents the specific clone and not a mixture of bands from the polyclonal amplification of normal B cells [13]. Even if polyacrylamide gels are used, it may be difficult to distinguish different clones by band size alone [10,14].

A single-strand conformation polymorphism (SSCP) analysis makes it possible to differentiate between the PCR products of equal size and differing in sequence by just one base [15]. When the PCR fragments of the clonal IgH chain rearrangements are denatured and electrophoresed under nondenaturing conditions at low temperatures, the single strands thus change into different conformations based on their unique sequences. Davis et al. utilized an SSCP analysis to detect clonal IgH chain gene rearrangements after PCR amplification of DNA from patients with B cell malignancies by using the consensus sequence primers of the VH and JH gene segments [16]. As a result, one malignant cell in 500 normal mononuclear cells (0.2%) was thus detected. However, because they used genomic DNA as the materials, their method provides no information about the isotype of the clone.

In this study, we combined the reverse transcriptase (RT)-PCR of the IgH chain transcripts with SSCP and analyzed the samples from B cell malignancies with the aim of developing a technique to analyze the clonal expansion of B cells. Because we used RNA as the materials instead of genomic DNA, we were thus able to determine the isotype of clonal populations as well as the presence of B cell clones and clonal identities of B cells from different samples.

TABLE I. Clinical Features

			Surface markers		
Patient	Age/sex	PBL count	smIg	CD5	CD19
CLL1	59/F	67000	μκ	—	+
CLL2	72/F	10800	μκ	+	+
CLL3	65/F	72690	μλ	+	+
CLL4	62/F	20060	ND[a]	+	+
CLL5	80/F	108940	μλ	+	+

	Age/sex	Stage	Status	Type	%PC
MM1	73/F	II	relapse	γκ	20.2%
MM2	62/M	IA	relapse	γλ	29.6%
MM3	76/F	IIIA	Untreated	γκ	58.8%
MM4	66/F	IIIA	Untreated	γλ	35.0%

			Surface markers	
	Age/sex	Serum IgM (g/dL)	smIg	CD19
WM1	57/F	12.0	μλ	+
WM2	70/M	8.5	μκ	+
WM3	72/M	1.8	ND[a]	ND

[a]Not done.

MATERIALS AND METHODS

Patients

Eight healthy subjects (N1 through N8), 5 B chronic lymphocytic leukemia (B-CLL) patients (CLL1 through CLL5), 4 multiple myeloma (MM) patients (MM1 through MM4) and 3 Waldenström's macroglobulinemia (WM) patients (WM1 through WM3) were studied (Table I). The diagnosis of B-CLL was made according to the criteria of the International Workshop on Chronic Lymphocytic Leukemia [17]. The tumor cells from all the B-CLL patients except for those from CLL1 expressed CD5 antigen. Monoclonal gammopathy and Bence-Jones proteinuria were absent in all of the B-CLL patients. The patients were diagnosed as having MM using the criteria of Durie and Salmon [18]. All of the WM patients were diagnosed according to the criteria of Mackenzie and Fudenberg [19]. Mononuclear cells (MNC) were isolated from heparinized peripheral blood (PB), pleural fluid and bone marrow (BM) by using the standard Ficoll/Hypaque gradient method. All specimens were obtained after receiving the patients written, informed consent.

RT-PCR

Total RNA from the cells was isolated by the previously described acid guanidinium thiocyanate-phenol-

TABLE II. Nucleotide Sequences of the Primers and Probes

Specificity	Sequence (5′–3′)
FR3 primer	ACACGGC(CT)(GC)TGTATTACTG
C_μ primer	GGAGAAAGTGATGGAGTCGGG
C_γ primer	GTAGTCCTTGACCAGGCAGC
C_α primer	TGAGTGGCTCCTGGGGGAAG
C_μ probe	AATTCTCACAGGAGACGAG
C_γ probe	AGGGGGAAGACCGATGGG
C_α probe	GCTCAGCGGGAAGACCTT
JH primer	CTGAGGAGACGGTGACC
JH probe	ACC(AG)(GT)(GT)GT(CT)CC(CT)(CT)GGCCCCAG

chloroform extraction method [20]. Total RNA (3μg) was converted into single-stranded cDNA by using random hexamer oligonucleotides and RT (Superscript, BRL, Gaithersburg, MD). The PCR was performed as described by Saiki et al. [12]. One μL of the RT reaction product was amplified using 20 pmole of VH FR3 and 1 of 3 constant region primers, and 2.5 U of Taq polymerase (Cetus, Norwalk, CT). A total of 35 cycles of amplification were performed (1 min at 94°C, 2 min at 54°C, 3 min at 72°C). To control for possible contamination, mock PCR reaction mixtures lacking template or containing products of the first strand cDNA reaction without RT were prepared. None of the controls contained amplified products visible on ethidium-stained agarose gels or product detected by SSCP analysis.

SSCP Analysis

The amplified DNA was diluted (1:20) in a denaturing solution (95% formamide, 10 mM ethylenediaminetetraacetic acid, 0.1% bromophenol blue, 0.1% xylene cyanol) and held at 90°C for 2 min. Two μL of the diluted sample was electrophoresed in nondenaturing 5% polyacrylamide gels containing 10% glycerol. The gel was run at 35 W constant power for about 2 hr. After electrophoresis, the DNA was transferred to Immobilon-S (Millipore Intertech, Bedford, MA) and then was incubated with a biotinylated internal constant region probe. The DNA was then visualized by subsequent incubations with streptavidin, biotinylated alkaline phosphatase and a chemilluminescent substrate system (Phototope detection kit, New England Biolabs, Beverly, MA).

Primers and Probes

The primers and probes used are described in Table II.

DNA Cloning and Sequencing

Slices containing the specific bands were excised from the polyacrylamide gel. The eluted DNA was then reamplified for 35 cycles with the same FR3 and constant region primers used in RT-PCR, except that they had a *Bam*HI and *Eco*RI cutting site, respectively, and were purified from 1.1% agarose gel. The recovered DNA fragments were ligated in the *Bam*HI/*Eco*RI site of pBluescript SK- (Stratagene, La Jolla, CA) and used to transform *Escherichia coli* strain XL-1Blue (Stratagene). The clones were picked randomly, and a double-stranded DNA template was prepared and sequenced by using the DyeDeoxy Terminator Cycle Sequencing kit and the Applied Biosystems automatic DNA sequencer (Applied Biosystems, Riossy, France).

PCR-SSCP Analysis to Analyze Clonal Relationship

To assess the potential relationship of the bands of different isotypes observed in individual B-CLL patient, slices containing the specific bands were excised from the polyacrylamide gel. The eluted DNA was reamplified for 35 cycles by using VH FR3 and a JH consensus primer. Amplified DNA was analyzed by SSCP as described above. Another internal JH consensus sequence was used as a detection probe.

RESULTS

To obtain information about the mRNA encoding the Ig H chain expressed in the selected population of human lymphocytes, the RT-PCR technique was used with a VH FR3 consensus sequence primer and IgH chain constant region primer. Ethidium bromide staining of conventional agarose gels showed the amplified DNA to have migrated as a single band (data not shown). Due to the fact that various diversities generated by the DH, JH, and N nucleotide sequences should be present within these bands, an attempt was thus made to discriminate these diversities by employing the SSCP technique, which has been reported to detect even a single nucleotide mutation. The RNA from PB MNC from healthy individuals developed as a smear following both RT-PCR and SSCP analyses (Fig. 1). This probably indicates an enomous degree of heterogeneity of the peripheral Ig H chain CDR3 repertoire. To confirm the specificity of constant region primers, the PCR product of each isotype was hybridized with constant region probes of different isotypes. In these experiments, no visible signals were observed (data not shown).

B-CLL usually involves the expansion of a clone of $CD5^+$ B cells synthesizing IgM antibodies [21]. Recent studies suggest that in some instances, terminal differentiation and isotype switching can occur both in vivo and in vitro [22–24]. To determine whether our RT-PCR-SSCP method can be applied for the detection of tumor clones and isotype switching, we analyzed the PB MNC from five cases of B-CLL (Fig. 2A). In patients CLL1 and CLL2, a single dominant band was detected in both IgM and IgA isotypes. In patient CLL3, dominant bands were detected in all three isotypes. In patients CLL4 and CLL5, two dominant bands in the IgG isotype and one dominant band in the IgM isotype were detected, respectively.

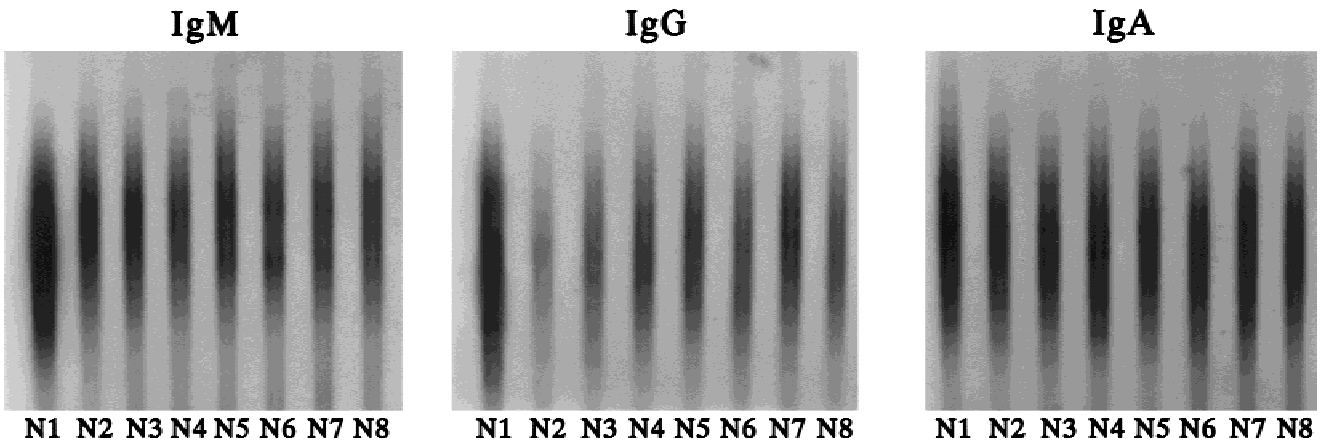
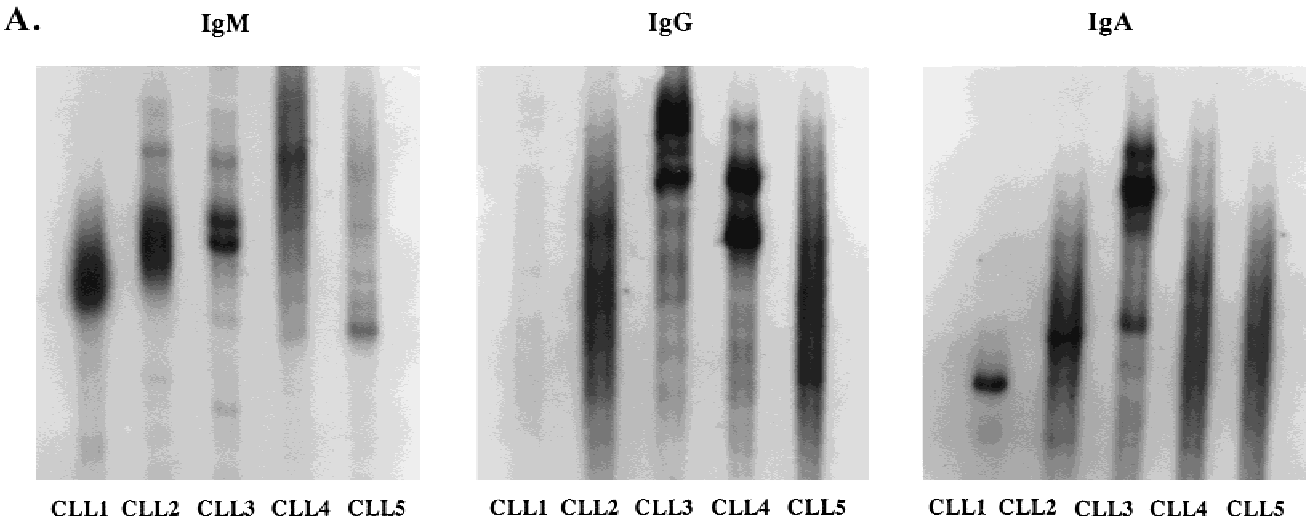


Fig. 1. Clonal analysis of the μ, γ, and α H chain messages of the PB from healthy subjects (N1–N8) using the RT-PCR-SSCP analysis. A typical smear pattern was observed in all healthy subjects.



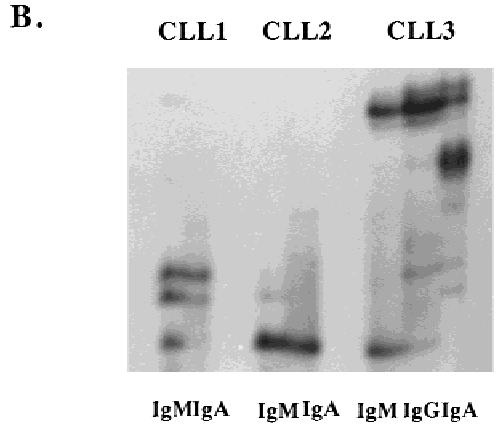


Fig. 2. A clonal analysis of the IgH chain messages of the PB from 5 B-CLL patients. A: The PB from CLL patients showed one or two dominant bands in at least one isotype. B: When dominant bands were observed in two or three isotypes in one individual, the clonal identities were then further analyzed as described in the Materials and Methods section.

In order to determine the relationship of dominant bands of different isotypes in each B-CLL individual, the eluted DNA from the specific bands were further analyzed by the PCR-SSCP method. Because we used consensus VH FR3 and JH sequences as upstream and downstream primers, respectively, for PCR and the internal JH consensus sequence as a detection probe, the transcripts of different isotypes which have an identical CDR3 sequense shoud show an identical migration following an SSCP analysis. The result of this analysis strongly sug-

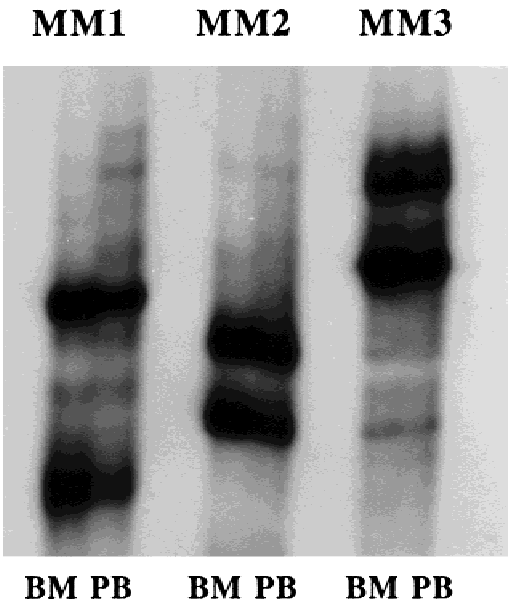


Fig. 3. An RT-PCR-SSCP analysis of the samples from the MM patients (MM1–MM3). Messages from the BM and PB of three MM patients were used. The PB from each patient showed two strong signals of an identical mobility with those from the BM in the IgG isotype.

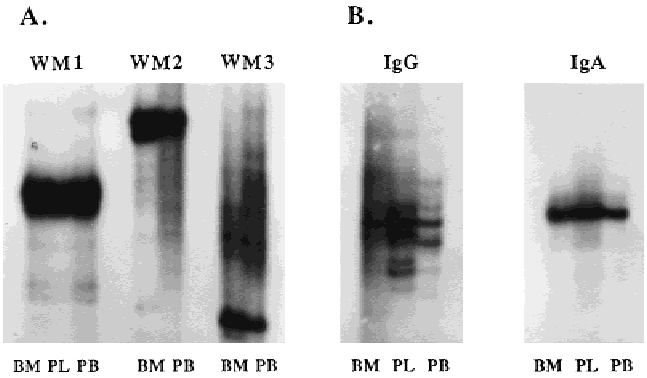


Fig. 4. A clonal analysis of the IgH chain messages from samples from WM patients (WM1–WM3). A: A single dominant band was observed in all samples from three WM patients in the IgM isotype. In each individual, the samples from the PB and BM showed an identical mobility. MNC from the pleural fluid (PL) which developed during the disease course of patient WM1 was also analyzed. B: In patient WM1, dominant bands were also observed in IgG and IgA isotypes.

gested that the dominant bands in the IgM and IgA isotypes in patients CLL1 and CLL2 had an identical CDR3 sequence. The dominant bands in all three isotypes in patient CLL3 also showed an identical migration pattern (Fig. 2B). We also sequence-analyzed the DNA from the dominant bands observed in the first RT-PCR-SSCP analysis and thus confirmed that the dominant bands of different isotypes in patients CLL1 and CLL2 had an identical CDR3 sequence (data not shown).

In a high proportion of MM patients, the monoclonal B cell population can often be detected in PB [25]. We analyzed four patients with MM whose PB lymphocytes were free from plasma cells by light microscopy. In three MM patients (patient MM1, MM2, and MM3), both BM and PB contained two dominant bands of the IgG isotype. The dominant bands were present at an identical position in BM and PB (Fig. 3). In patient MM4, we could not observe any dominant bands (data not shown).

With respect to patients MM1 and MM2, we sequence-analyzed all dominant bands. We thus found that the two dominant IgG bands in each lane had an identical sequence, suggesting that these two bands resulted from a different mobilization of the single PCR product from a single clone. Dominant bands of an identical mobility in BM and PB in each patient also had an identical CDR3 sequence (data not shown).

WM is a malignant lymphoplasmo-proliferative disorder with monoclonal pentameric IgM production. The most consistent feature of the clonal B cells in the BM and/or lymph node of patients with WM is pleomorphic B lineage cells at different stages of maturation, such as small lymphocytes, lymphoplasmacytoid cells, and plasma cells [26]. In WM1 and WM3, 7% and 3% of leukocytes in PB were abnormal lymphoid cells, respectively. In WM2, we could not detect any abnormal cells in PB by morphologic and flowcytometric techniques. To determine whether or not a clonal B cell population is present in the PB from WM and to also analyze the relationship between clones from BM and PB, we analyzed the MNC from BM and PB from three WM patients (WM1, WM2, and WM3) using the RT-PCR-SSCP method (Fig. 4A). A single dominant band of the IgM isotype which was common in BM and PB was observed in all three patients. In addition to IgM monoclonal protein, trace amounts of monoclonal IgG and IgA protein were also detected in patient WM1. In this patient, dominant bands were observed in three isotypes (Fig. 4A,B). Harboe et al. reported that 14% of the sera from patients with WM contained more than one monoclonal Ig [27]. However, as to whether or not these clones were clonally related, no definite conclusions could be made. We performed a sequence analysis of the dominant bands of three isotypes in RT-PCR-SSCP analysis of PB, and found them to have an identical CDR3 sequence (data not shown).

During the course of the disease, patient WM1 developed pleural effusion. A histological analysis revealed that 90% of cells in the pleural fluid were plasma cells. The rest of the cells included macrophages and mesothelial cells. To identify the origin of the plasma cells, we performed an RT-PCR-SSCP analysis using the cells from the pleural fluid. The dominant band showed similar migration pattern with those of the monoclonal cells from BM and PB (Fig. 4A,B), which showed that the

plasma cells in pleural fluid were derived from the clone in BM or PB. The sequence analysis of the CDR3 region of dominant bands of the IgM isotype from BM, PB, and pleural fluid confirmed this result (data not shown).

DISCUSSION

As was expected due to the enormous diversity of the IgH CDR3 region, an analysis of B cells in the PB from healthy subjects exhibited a smear-like pattern which appeared to be a summation of multiple bands with heterogenous mobilities. The IgH chain CDR3 region is important in that it forms the center of the antigen binding site [6] and contributes to the generation of diversity in the pre-immune H chain repertoire through the rearrangement of VH, DH, and JH gene rearrangement and the addition of the N regions [1,2]. Our method analyzes the sequence difference in this CDR3 region. If the smear-like pattern observed in healthy individuals reflects the heterogenous repertoire which is essential to specific immunity, then our method may be useful for evaluating the immune status.

Most patients with B-CLL have clonal amplifications of IgM^+ B lymphocytes that are blocked at the surface membrane Ig-expressing stage of B cell maturation [21]. However, in vitro studies have suggested that B-CLL cells are not permanently frozen at this stage of differentiation, because appropriate stimulation can give rise to terminal differentiation and to isotype switching [23]. Previous studies of patient samples also support the notion that these processes can occur in vivo [22–24]. In three of five B-CLL patients (CLL1, CLL2, and CLL3), multiple isotype transcripts were observed (Fig. 2A,B). These findings are compatible with previous reports. To the best of our knowledge, CLL1 is the first known $CD5^-$ CLL patient who showed multiple isotype transcripts.

When the monoclonal Igs of different isotypes were detected in individual samples, we analyzed the relationship of each monoclonal band by excising the band and then further analyzing it by a PCR-SSCP analysis using FR3 and JH consensus sequence primers (Fig. 2B). This is a useful method to analyze the clonal relationship when multiple clones of different isotypes were detected in individual patients.

Multiple isotype transcripts should be the products of isotype switching or differential RNA splicing. Immunohistochemistry is not sifficient to discriminate these two mechanisms, because all transcripts may not be functional templates for protein synthesis and also expression of each Ig isotype at low level or in minor cell populations may be interfered by serum Ig. To detect subpopulations of tumor cells that may have undergone deletional isotype switch, expression of each Ig needs to be studied at the single-cell level.

MM is a malignant tumor involving plasma cells that accumulate in the BM [28]. It has been shown that malignant cells circulate in the blood of most patients with active MM [25,29–32]. Our RT-PCR-SSCP method was able to detect the presence of common clonal B cell expansion in PB and BM (Fig. 3). Unique feature of SSCP also enables us to easily demonstrate the clonal identities of expanded B cell populations in PB and BM.

In patients MM1, MM2, and MM3, two dominant bands of the IgG isotype were detected in each analysis (Fig. 3). With respect to patients MM1 and MM2, we sequenced the two dominant bands and confirmed that they had an identical CDR3 sequence (data not shown). It is well known that a particular single-stranded DNA can take at least two different molecular shapes, depending on the conditions of electrophoresis [15]. By adjusting the condition of electrophoresis, especially the temperature of the running gels, we may obtain a single dominant band. In order to know whether the two dominant bands have identical sequence or not, we can apply the method that we used to analyze the relationship of monoclonal Igs of different isotypes in individual CLL samples (Fig. 2B).

Several observations have suggested that monoclonal B cell populations might be present in the PB of WM [26,33–35]. However, there have been no studies which directly examined the presence of clonal B cells in PB and also analyzed the clonal relationship of B cell clones from BM and PB by using molecular techniques. This is important because WM is extremely pleomorphic with respect to its tumor clones. Our study demonstrated that the dominant B cell clone in BM was also detectable in the PB of WM patients and multiple isotype transcripts could be observed in WM (Fig. 4A,B). A strong dominant band was detected in PB of WM2 where no abnormal cells were detected by morphologic and flowcytometric techniques. The detection of tumor clones in the PB is important when we consider the treatment of this malignant disease because its presence in the PB is considered to result in a poor prognosis [35].

Patient WM1 developed pleural effusion in her clinical course. An RT-PCR-SSCP analysis clearly demonstrated that the origin of plasma cells in the pleural fluid was the monoclonal cells found in BM and PB (Fig. 4A,B). Our analysis is useful for analyzing the nature of lesions which newly developed during the course of the disease, such as WM, whose tumor cells are pleomorphic and thus the histogy of the new lesions may be quite different.

As a universal 5′ primer, we used the conserved consensus sequence present at the 3′ end of VH FR3 region. Under the right annealing conditions, this oligonucleotide is able to hybridize to more than 95% of the published VH sequences irrespective of the family to which they belong [7]. In the T cell-dependent immune response, however, B cells undergo a somatic mutation of their Ig V genes, thus resulting in a decrease in the rate

of hybridization of the VH FR3 primer to somatically mutated VH genes. The hybridization rate varies between about 10% to 100% depending on the kinds of B cell malignancies [36]. VH gene use in B-CLL is highly restricted [37,38] and the V region sequences in this disease are usually unmutated [39–41]. Although isotype switching in B-CLL has been noted, it does not necessarily accompany the affinity maturation and selection [23,24]. Thus, B-CLL appears to be appropriate for the RT-PCR-SSCP method. In fact, in five of five B-CLL patients, we could detect at least one clonal band. The majority of V genes in MM appear to be heavily mutated in keeping with the mature B cell derivation of this malignancy [42]. Both an idiotypic and sequence analysis suggest that the VH gene repertoire of this mature B cell malignancy is restricted [42,43] and an analysis of the tumor-derived V genes indicates that there is little intraclonal variation or ongoing somatic mutation [42,44]. Our method was not able to detect clonal bands in one of four MM patients. However, these findings support that once a clonal population can be detected, then our method is useful for monitoring minimal residual disease in MM after treatment. Aoki et al. performed a DNA analysis of the Ig V gene CDR3 of the tumor cells in WM and found replacement mutations in the DH and/or JH segments in all patients but no intraclonal variations resulting from the VH replacements or ongoing somatic mutation [45]. Therefore, the RT-PCR-SSCP method appears to also be useful in the analysis of tumor cells in WM.

Serial dilution experiments, in which the MNC from CLL1 was serially diluted into the MNC from a healthy subject, revealed that the RT-PCR-SSCP analysis could detect one malignant cell in 100 MNC (data not shown). This result is similar to that by Davis et al. (0.2%) [16]. By using BM and PB MNC from a Sjögren's syndrome patient with μλ paraprotein, we performed the RT-PCR-SSCP analysis and Southern analysis. Although no IgH chain gene rearrangement was observed in Southern analysis, the RT-PCR-SSCP analysis could detect a clear single band in both BM and PB samples (data not shown). Thus, the sensitivity of the RT-PCR-SSCP analysis is better than that of Southern analysis. Because FR3 consensus primer is not clone specific, PCR amplifies all CDR3 regions in the sample. In order to avoid false-positive interpretation of minor reactive populations as malignant clones, the sensitivity of the RT-PCR-SSCP analysis appears adequate. However, the sensitivity should be quite different depending on the sequence of the consensus sequence portion of the VH gene of the clone.

As far as we performed sequence analyses, SSCP bands of an identical mobility showed an identical sequence. This does not mean that bands of the same mobility always have completely identical sequences. Minor sequence differences may not produce different mobility bands depending on the conditions of SSCP. In order to study the questions of clonal development and diversification in detail, we should use the RT-PCR-SSCP analysis in conjunction with clone specific primer or probe-based methods and sequence analysis.

CONCLUSIONS

We combined the RT-PCR of the IgH chain transcripts with SSCP and analyzed the samples from B cell malignancies. Because we used RNA as the materials instead of genomic DNA, we were thus able to determine the isotype of clonal populations as well as the presence of B cell clones and clonal identities of B cells from different samples. Tumor-related clones in PB were detected by an RT-PCR-SSCP analysis in MM and WM and clonal identities of B cell clones in PB and BM were also easily demonstrated by the similar mobility on SSCP gel. Although the number of the patients analyzed in our study was small, presence of multiple isotype transcripts appeared to be frequent in B cell malignancies.

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